

FORM PTO-1390 (Modified) (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>RTSP-0153</b>
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR <b>09/937646</b>
INTERNATIONAL APPLICATION NO. <b>PCT/US00/07634</b>	INTERNATIONAL FILING DATE <b>23 March 2000</b>	PRIORITY DATE CLAIMED <b>29 March 1999</b>		
TITLE OF INVENTION <b>Antisense Modulation of SRA Expression</b>				
APPLICANT(S) FOR DO/EO/US <b>COWSERT, Lex M. et al.</b>				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2))        a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).        b. <input type="checkbox"/> has been communicated by the International Bureau.        c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).        a. <input type="checkbox"/> is attached hereto.        b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))        a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).        b. <input type="checkbox"/> have been communicated by the International Bureau.        c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.        d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</p> <p>11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409).</p> <p>12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210).</p>				
<b>Items 13 to 20 below concern document(s) or information included:</b>				
<p>13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>15. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>16. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>17. <input type="checkbox"/> A substitute specification.</p> <p>18. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>19. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with 37 CFR 1.821-1.825 and amended sequence list and diskette containing computer readable copy of Sequence Listing;</p> <p>20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 371.</p> <p>21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 371.</p> <p>22. <input type="checkbox"/> Certificate of Mailing by Express Mail</p> <p>23. <input checked="" type="checkbox"/> Other items or information:</p>				
<p>1) Courtesy copy of International Application</p> <p>2) Statement to Support Filing and Submission in Accordance with 37 CFR 1.821-1.825 and amended sequence list and diskette containing computer readable copy of Sequence Listing;</p> <p>3) Return post card</p>				
<p>"Express Mail" Label No. <b>EL750774475US</b> Date of Deposit <b>September 27, 2001</b></p> <p>I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.</p> <p>By <u>Deborah Ehret</u> Typed Name: Deborah Ehret</p>				

U.S. APPLICATION NO. (IF KNOWN SEE 37 CFR <b>09/937646</b>	INTERNATIONAL APPLICATION NO. PCT/US00/07634	ATTORNEY'S DOCKET NUMBER RTSP-0153																
24. The following fees are submitted:		<b>CALCULATIONS PTO USE ONLY</b>																
<b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</b>																		
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b> <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b>																		
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		<b>\$860.00</b>																
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).		<input type="checkbox"/> 20 <input type="checkbox"/> 30 <b>\$0.00</b>																
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 25%;">CLAIMS</th> <th style="width: 25%;">NUMBER FILED</th> <th style="width: 25%;">NUMBER EXTRA</th> <th style="width: 25%;">RATE</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>17 - 20 =</td> <td>0</td> <td>x \$18.00      <b>\$0.00</b></td> </tr> <tr> <td>Independent claims</td> <td>1 - 3 =</td> <td>0</td> <td>x \$80.00      <b>\$0.00</b></td> </tr> <tr> <td colspan="2">Multiple Dependent Claims (check if applicable).</td> <td style="text-align: center;"><input type="checkbox"/></td> <td style="text-align: center;"><b>\$0.00</b></td> </tr> </tbody> </table>		CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	Total claims	17 - 20 =	0	x \$18.00 <b>\$0.00</b>	Independent claims	1 - 3 =	0	x \$80.00 <b>\$0.00</b>	Multiple Dependent Claims (check if applicable).		<input type="checkbox"/>	<b>\$0.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE															
Total claims	17 - 20 =	0	x \$18.00 <b>\$0.00</b>															
Independent claims	1 - 3 =	0	x \$80.00 <b>\$0.00</b>															
Multiple Dependent Claims (check if applicable).		<input type="checkbox"/>	<b>\$0.00</b>															
<b>TOTAL OF ABOVE CALCULATIONS =</b>		<b>\$860.00</b>																
<input checked="" type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.		<b>\$430.00</b>																
<b>SUBTOTAL =</b>		<b>\$430.00</b>																
Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).		<input type="checkbox"/> 20 <input type="checkbox"/> 30      + <b>\$0.00</b>																
<b>TOTAL NATIONAL FEE =</b>		<b>\$430.00</b>																
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).		<input type="checkbox"/> <b>\$0.00</b>																
<b>TOTAL FEES ENCLOSED =</b>		<b>\$430.00</b>																
<b>Applicant, ISIS Pharmaceuticals, Inc. is entitled to small entity status. It is a small business concern.</b>		<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%;">Amount to be: refunded</td> <td style="width: 50%;">\$</td> </tr> <tr> <td>charged</td> <td>\$</td> </tr> </table>	Amount to be: refunded	\$	charged	\$												
Amount to be: refunded	\$																	
charged	\$																	
a. <input checked="" type="checkbox"/> A check in the amount of <b>\$430.00</b> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <b>50-1619</b> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.																		
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>																		
<b>SEND ALL CORRESPONDENCE TO:</b> <div style="border: 1px solid black; padding: 5px; width: fit-content;">           Jane Massey Licata, Reg. No. 32,257            Kathleen A. Tyrrell, Reg. No. 38,350            Licata &amp; Tyrrell P.C.            66 E. Main Street            Marlton, New Jersey 08053             Telephone:(856) 810-1515            Facsimile:(856) 810-1454         </div> <div style="margin-top: 10px;">             SIGNATURE  <hr/>           Kathleen A. Tyrrell            NAME  <hr/>           38,350            REGISTRATION NUMBER  <hr/>           September 27, 2001            DATE         </div>																		

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: RTSP-0153  
Inventors: Cowser et al.  
Serial No.: Not yet assigned  
Filing Date: Not yet assigned  
Examiner: Not yet assigned  
Group Art Unit: Not yet assigned  
Title: Antisense Modulation of SRA Expression

"Express Mail" Label No. EL 750774475 US  
Date of Deposit - September 27, 2001

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By Deborah Ehret  
Typed Name: Deborah Ehret

BOX SEQUENCE  
Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

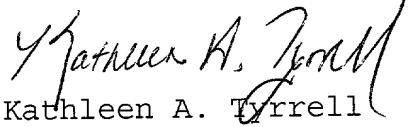
## PRELIMINARY AMENDMENT

It is respectfully requested that the Sequence Listing of the instant specification be deleted and replaced with the amended Sequence Listing provided herewith. A paper copy and a CRF copy of the amended Sequence Listing are provided herewith.

The replacement Sequence Listing has been amended to conform with the current Sequence Listing Rules. However, data presented in the Sequence Listing is identical to that originally filed.

Thus, no new matter has been added by this amendment.

Respectfully submitted,

  
Kathleen A. Tyrrell  
Registration No. 38,350

Date: September 27, 2001

LICATA & TYRRELL P.C.  
66 E. Main Street  
Marlton, New Jersey 08053

(856) 810-1515

## SEQUENCE LISTING

<110> Lex M. Cowser  
 C. Frank Bennett  
 Bert W. O'Malley

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Dose-Response Effects

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PTO/PGT Rec'd 27 SEP 2001

## ANTISENSE MODULATION OF SRA EXPRESSION

## FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of SRA. In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with SRA or nucleic acids encoding human SRA. Such oligonucleotides have been shown to modulate the expression of SRA.

## 10 BACKGROUND OF THE INVENTION

Steroid, thyroid and retinoid hormones produce a diverse array of physiologic effects through the regulation of gene expression. Upon entering the cell, these hormones bind to a unique group of intracellular nuclear receptors which have been characterized as ligand-dependent transcription factors. This complex then moves into the nucleus where the receptor and its cognate ligand interact with the transcription preinitiation complex affecting its stability and ultimately the rate of transcription of the target genes.

The interactions of the liganded receptor with the specific elements in the promoter region of the target genes are mediated by multi-protein complexes. These complexes are composed of either corepressors, which inhibit transactivation or coactivators, which enhance transactivation (Glass et al., *Curr. Opin. Cell. Biol.*, 1997, 9, 222-232). Several nuclear receptor coactivator proteins have been identified, all having similar biochemical properties. Recently, however, a novel coactivator was reported to be the first endogenous RNA transcript to function as an eukaryotic transcriptional coactivator. This coactivator, SRA, or steroid receptor RNA activator, has several unique features. SRA selectively interacts with the AF-1 domain of steroid

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hormone receptors found in the amino terminus of the receptor. Nuclear receptors contain two AF (activation function) domains responsible for their transcription activation function, and previously identified 5 coactivators have all been shown to interact through the AF-2 domain.

SRA is also expressed as multiple isoforms in a tissue and cell-specific manner. Multiple transcripts of SRA were isolated and fell into two categories; a major transcript 10 fraction of 0.7-0.85 kilobases (kb) in length producing a doublet and a minor transcript fraction of 1.3-1.5 kilobases. Of the two major transcripts in the doublet, the 0.7 kb species was expressed at significantly higher levels in breast cancer cell lines. All of the transcripts were 15 found to exist at different levels in the tissues examined but were enriched in the liver and skeletal muscle.

Finally, SRA was found to be present in a preformed coregulator complex with the AF-2 coactivator, SRC-1. SRC-1 or steroid receptor coactivator-1 has been shown to 20 enhance the transcriptional activity of the estrogen, glucocorticoid, thyroid, progesterone and retinoic acid receptors (Onate et al., *Science*, 1995, 270, 1354-1357). In immunoprecipitation experiments, it was shown that SRA acts as a component of distinct ribonucleoprotein 25 complexes, one of which contains the nuclear receptor coactivator, SRC-1. Thus the SRA mRNA transcript itself, specifically expressed in steroid target tissues, functions as a component of a large multiprotein complex to selectively enhance transcriptional activation by steroid 30 receptors.

Currently, there are no known therapeutic agents that effectively inhibit the synthesis of SRA. Consequently, there remains a long felt need for agents capable of selectively and effectively inhibiting SRA function.

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Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the  
5 modulation of SRA expression.

#### **SUMMARY OF THE INVENTION**

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to SRA or a nucleic acid encoding SRA, and which  
10 modulate SRA or the expression of SRA. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating levels of SRA in cells or tissues comprising contacting said cells or tissues with one or  
15 more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with SRA by administering a therapeutically or prophylactically  
20 effective amount of one or more of the antisense compounds or compositions of the invention.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in  
25 modulating the function of SRA or nucleic acid molecules encoding SRA, ultimately modulating the amount of SRA present. This is accomplished by providing antisense compounds which specifically hybridize with an SRA transcript or nucleic acids encoding SRA. As used herein,  
30 the terms "target nucleic acid" and "nucleic acid encoding SRA" encompass SRA, DNA encoding SRA, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes  
35 with the normal function of the nucleic acid. This

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modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The 5 functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or 10 facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of SRA. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression 15 of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target. Because it is presently believed that SRA may not be translated and the SRA mRNA transcript itself may be the functional moiety in 20 transcriptional coactivation, the target molecule is the SRA mRNA transcript itself, which may then be inactivated or degraded through antisense activity, or a nucleic acid encoding SRA, antisense targeting to which would then block SRA expression.

25 It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose 30 function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is 35 SRA or a nucleic acid molecule encoding SRA. The targeting

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process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons, if any, that would be used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding SRA, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and

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"translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly,

5 the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

10 The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5'

15 untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on

20 the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on  
25 the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap.

30 The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated)  
35 regions are known as "exons" and are spliced together to

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form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an 5 overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds 10 targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired 15 effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, 20 adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is 25 capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a 30 sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing 35 such that stable and specific binding occurs between the

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oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

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In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides 5 composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms 10 because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form 15 of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 20 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 linked nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside 25 is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that 30 include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form 35 a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to

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form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the  
5 oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural  
10 internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as  
15 sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates,  
20 phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates,  
25 thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to  
30 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808;  
35 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897;

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5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;  
5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;  
5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;  
5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of  
5 which are commonly owned with this application, and each of  
which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl  
10 internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside);  
15 siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide  
20 backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 25 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of  
30 which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups.  
35 The base units are maintained for hybridization with an

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- appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.
- 5 Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 10 15 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH<sub>2</sub>-NH-O-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-O-CH<sub>2</sub>- [known as a 20 methylene (methylimino) or MMI backbone], -CH<sub>2</sub>-O-N(CH<sub>3</sub>)-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-CH<sub>2</sub>- and -O-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>- [wherein the native phosphodiester backbone is represented as -O-P-O-CH<sub>2</sub>-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 25 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides 30 comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are 35 O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>,

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and  $O(CH_2)_nON[(CH_2)_mCH_3)]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl,

substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-

5 aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>,

ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl,

aminoalkylamino, polyalkylamino, substituted silyl, an RNA

cleaving group, a reporter group, an intercalator, a group

for improving the pharmacokinetic properties of an

10 oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al.,

15 *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoxyethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-

20 O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3'

position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5'

25 terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:

30 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;

5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;

5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;

5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920,

certain of which are commonly owned with the instant

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application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines,

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including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., 5 *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 15 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the 20 instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 25 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic

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chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 20 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 25 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 30 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than

one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds.

- 5 "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an
- 10 oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target
- 15 nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results
- 20 in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate
- 25 deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.
- 30 Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to
- 35 in the art as hybrids or gapmers. Representative United

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States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.:  
5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;  
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;  
5 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through  
10 the well-known technique of solid phase synthesis.

Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well  
15 known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector  
20 constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as  
25 for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting  
30 formulations include, but are not limited to, U.S.:  
5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;  
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;  
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;  
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;  
35 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;

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5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et

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al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt 5 in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility 10 in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of 15 the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and 20 include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for 25 example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, 30 mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis 35 of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid,

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methanesulfonic acid, ethanesulfonic acid,  
2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid,  
benzenesulfonic acid, 4-methylbenzenesulfoic acid,  
naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic  
5 acid, 2- or 3-phosphoglycerate, glucose-6-phosphate,  
N-cyclohexylsulfamic acid (with the formation of  
cyclamates), or with other acid organic compounds, such as  
ascorbic acid. Pharmaceutically acceptable salts of  
compounds may also be prepared with a pharmaceutically  
10 acceptable cation. Suitable pharmaceutically acceptable  
cations are well known to those skilled in the art and  
include alkaline, alkaline earth, ammonium and quaternary  
ammonium cations. Carbonates or hydrogen carbonates are  
also possible.

15 For oligonucleotides, preferred examples of  
pharmaceutically acceptable salts include but are not  
limited to (a) salts formed with cations such as sodium,  
potassium, ammonium, magnesium, calcium, polyamines such as  
spermine and spermidine, etc.; (b) acid addition salts  
20 formed with inorganic acids, for example hydrochloric acid,  
hydrobromic acid, sulfuric acid, phosphoric acid, nitric  
acid and the like; (c) salts formed with organic acids  
such as, for example, acetic acid, oxalic acid, tartaric  
acid, succinic acid, maleic acid, fumaric acid, gluconic  
25 acid, citric acid, malic acid, ascorbic acid, benzoic acid,  
tannic acid, palmitic acid, alginic acid, polyglutamic  
acid, naphthalenesulfonic acid, methanesulfonic acid,  
p-toluenesulfonic acid, naphthalenedisulfonic acid,  
polygalacturonic acid, and the like; and (d) salts formed  
30 from elemental anions such as chlorine, bromine, and  
iodine.

The antisense compounds of the present invention can  
be utilized for diagnostics, therapeutics, prophylaxis and  
as research reagents and kits. For therapeutics, an  
35 animal, preferably a human, suspected of having a disease

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or disorder which can be treated by modulating the expression of SRA is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to SRA or nucleic acids encoding SRA, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with SRA or a nucleic acid encoding SRA can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of SRA in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial,

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subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be  
5 particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical  
10 carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in  
15 water or non-aqueous media, capsules, sachets or tablets.

Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include  
20 sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention  
25 include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

30 The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association  
35 the active ingredients with the pharmaceutical carrier(s)

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or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the  
5 product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the  
10 present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The  
15 suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams,  
20 jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may  
25 be applied to the formulation of the compositions of the present invention.

#### Emulsions

The compositions of the present invention may be  
30 prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in

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Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

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Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion.

Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger,  
20 in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are  
25 typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the  
30 preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*,

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Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, 5 lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers 10 especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium 15 aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, 20 fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage 25 Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, 30 carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form 35 colloidal solutions that stabilize emulsions by forming

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strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

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In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245;

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Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of  
5 solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-  
10 ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate  
15 (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into  
20 the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art.  
25 The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355,  
30 Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

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Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral

- 5 bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from
- 10 enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity
- 15 (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile
- 20 drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will
- 25 facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity
- 30 and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and

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to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

10 **Liposomes**

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

20 Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-25 cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each 30 with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes 35 obtained from natural phospholipids are biocompatible and

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biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

10        Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As 15 the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

10        Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many 20 drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the 25 administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

10        Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into 30 the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

15        Liposomes fall into two broad classes. Cationic 35 liposomes are positively charged liposomes which interact

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with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, 5 the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it.

10 Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene 15 to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived

20 phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are 25 formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

30 Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an 35 emulsion) were ineffective (Weiner et al., *Journal of Drug*

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Targeting, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal 5 formulation was superior to aqueous administration (du Plessis et al., Antiviral Research, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant 10 and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to 15 deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. S.T.P.Pharma. Sci., 1994, 4, 6, 466).

20 Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such 25 specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as 30 a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these 35 sterically stabilized liposomes derives from a reduced

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uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art.

- 5 Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G<sub>M1</sub>, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G<sub>M1</sub> or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycals results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycals (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended

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such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG.

Liposomes having covalently bound PEG moieties on their

5 external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633)

10 and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*)

15 Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

20 A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense 30 oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly

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deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of 5 pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum 10 albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such 15 as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also 20 known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is 25 classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic 30 esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block 35 polymers are also included in this class. The

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polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant 5 is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and 10 sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant 15 is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry 20 either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations 25 and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

#### Penetration Enhancers

In one embodiment, the present invention employs 30 various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell 35 membranes. It has been discovered that even non-lipophilic

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drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance 5 the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic 10 Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical 15 entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In 20 addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical 25 emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), 30 myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines,

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$C_{1-10}$  alkyl esters thereof (e.g., methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in*

- 5 *Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

Bile salts: The physiological role of bile includes

- 10 the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic  
15 derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable  
20 sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycocodeoxycholic acid (sodium glycocodeoxycholate), taurocholic acid (sodium taurocholate),  
25 taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydrofusidate (STDHF), sodium glycidihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical  
30 Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33;

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Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25;  
Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac

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sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

#### Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-

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4'-isothiocyanostilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

**Excipients**

5 In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is  
10 selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to,  
15 binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen  
20 phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch  
25 glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be  
30 used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin,

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hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as 5 alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously 10 react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, 15 hydroxymethylcellulose, polyvinylpyrrolidone and the like.

#### Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at 20 their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional 25 materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere 30 with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic 35 pressure, buffers, colorings, flavorings and/or aromatic

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substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for 5 example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other 10 chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, 15 cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUDR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual 20 of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflamatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, 25 acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents 30 are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic 35 acid and one or more additional antisense compounds

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targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

**EXAMPLES****Example 1****Nucleoside Phosphoramidites for Oligonucleotide Synthesis****5 Deoxy and 2'-alkoxy amidites**

2'-Deoxy and 2'-methoxy beta-cyanoethylisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are 10 prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was 15 increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available 20 phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

**2'-Fluoro amidites****2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as 25 described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D- 30 arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S<sub>N</sub>2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-

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ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-

5 phosphoramidite intermediates.

#### **2'-Fluorodeoxyguanosine**

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

#### **2'-Fluorouridine**

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3' phosphoramidites.

#### **2'-Fluorodeoxycytidine**

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3' phosphoramidites.

#### **2'-O-(2-Methoxyethyl) modified amidites**

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods

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of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

**2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

5-Methyluridine (ribosylthymine, commercially

5 available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner.  
10 After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 15 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the 20 structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

25       **2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating 30 for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was 35 dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel

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column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub>, and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the

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addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-

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dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL).

- 5 The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue  
10 was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

- 15 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the  
20 reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96  
25 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

- 30 N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with  
35 stirring, under a nitrogen atmosphere. The resulting

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mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and the extracts were combined, dried over MgSO<sub>4</sub> and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

**2'-(Dimethylaminooxyethoxy) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

**5'-O-tert-Butyldiphenylsilyl- $O^2$ -2'-anhydro-5-methyluridine**

25 O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium

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bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the  
5 solution was cooled to

-10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

10        5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene  
15 glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-  
Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g,  
0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were  
added with manual stirring. The reactor was sealed and  
20 heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70%  
25 conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once  
30 the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate  
35 fractions were combined, stripped and dried to product as a

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white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99%  
5 pure product.

**2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-  
5-methyluridine**

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with  
10 triphenylphosphine (11.63g, 44.36mmol) and N-hydroxypthalimide (7.24g, 44.36mmol). It was then dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a  
15 clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred  
20 for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-  
25 butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

**5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-  
30 5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH<sub>2</sub>Cl<sub>2</sub> and the combined organic phase was washed with water, brine and

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dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to get 2'-O-(aminoxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

**5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-**

**dimethylaminoxyethyl]-5-methyluridine**

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness . The residue obtained was

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purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>, to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

5       **2'-O-(dimethylaminoxyethyl)-5-methyluridine**

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>, to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

15      **5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine**

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.13g, 80%).

25      **5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-**

30      **methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g,

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1.67mmol) was added and dried over  $P_2O_5$  under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) 5 was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-10 2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam 15 (1.04g, 74.9%).

**2'-(Aminooxyethoxy) nucleoside amidites**

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs.

20 Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

**N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

25 The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinossso, C. J., WO 94/02501 A1 940203.) Standard protection procedures 30 35 should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-

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dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

**Example 2**

**Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester ( $P=O$ ) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates ( $P=S$ ) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or

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5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

5 Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

10 3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

15 Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

**Example 3**

**Oligonucleoside Synthesis**

20 Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 25 5,489,677, 5,602,240 and 5,610,289, all of which are 30 herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

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Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

**Example 4**

**5 PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 10 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

**Example 5**

**Synthesis of Chimeric Oligonucleotides**

15 Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked 20 nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are 25 also known in the art as "hemimers" or "wingmers".

**[2'-O-Me] -- [2'-deoxy] -- [2'-O-Me] Chimeric**

**Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo-30 nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-

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methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The 5 fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect 10 all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 15 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate**

20 **Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites. 25

**[2'-O-(2-Methoxyethyl) Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides**

[2'-O-(2-methoxyethyl) phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above 30 procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate

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the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3, H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide  
5 linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

10 **Example 6**

**Oligonucleotide Isolation**

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the  
15 oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The  
20 relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by  $^{31}\text{P}$  nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* 1991, 266,  
25 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

**Example 7**

**Oligonucleotide Synthesis - 96 Well Plate Format**

30 Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format.

Phosphodiester internucleotide linkages were afforded by

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oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-

- 5 protected beta-cyanoethylisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base
- 10 protected beta-cyanoethylisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH<sub>4</sub>OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in  
15 sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

**Example 8**

**Oligonucleotide Analysis - 96 Well Plate Format**

20 The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for  
25 individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and  
30 multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

**Example 9**

**Cell culture and oligonucleotide treatment**

35 The effect of antisense compounds on target nucleic

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acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 four cell types are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells:

The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection

10 (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 15 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

20 For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

25 The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life 30 Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

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NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds:

When cells reached 80% confluence, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

**Example 10**

**Analysis of oligonucleotide inhibition of SRA expression**

Antisense modulation of SRA or SRA expression can be assayed in a variety of ways known in the art. For example, SRA mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example,

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Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel,

- 5 F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems,  
10 Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

It is presently believed that SRA is not translated to a protein. However, if appropriate, protein levels can be  
15 quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al.,  
20 *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997.  
Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley  
25 & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and

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can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

**Example 11**

5 **Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

**Example 12**

**Total RNA Isolation**

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following

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the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 100  $\mu$ L Buffer RLT was added to each well and the plate 5 vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96<sup>TM</sup> well plate attached to a QIAVAC<sup>TM</sup> manifold fitted with a waste collection tray and 10 attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96<sup>TM</sup> plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96<sup>TM</sup> plate and the vacuum applied for a period of 15 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC<sup>TM</sup> manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC<sup>TM</sup> manifold fitted with a collection tube rack 20 containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60  $\mu$ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60  $\mu$ L water.

**Example 13**

25 **Real-time Quantitative PCR Analysis of SRA mRNA Levels**

Quantitation of SRA mRNA levels was determined by real-time quantitative PCR using the ABI PRISM<sup>TM</sup> 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This 30 is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in 35 real-time quantitative PCR are quantitated as they

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accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, 5 obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to 10 the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq 15 polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With 20 each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel 25 reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, 30 Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl<sub>2</sub>, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 35 12.5 Units MuLV reverse transcriptase) to 96 well plates

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containing 25  $\mu$ L poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C.

Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were

5 carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). SRA probes and primers were designed to hybridize to a consensus region of the four human SRA sequences against which antisense oligonucleotides were designed, incorporated herein as SEQ

10 ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4).

For SRA the PCR primers were:

forward primer: GTGGCCACACAAAGGAAGCA (SEQ ID NO: 5)

reverse primer: CTTTCCTCCAGCCCCTGTTC (SEQ ID NO: 6) and the PCR probe was: FAM-ATGACATCAGCCGACGCCTGGA-TAMRA

15 (SEQ ID NO: 7) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 8)

20 reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 9) and the PCR probe was: 5' JOE-CAAGCTTCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 10) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

25 **Example 14**

**Northern blot analysis of SRA mRNA levels**

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA 30 was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ 35 nylon membranes (Amersham Pharmacia Biotech, Piscataway,

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NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking  
5 using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA).

Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a SRA  
10 specific probe prepared by PCR using the forward primer GTGGCCACACAAGGAAGCA (SEQ ID NO: 5) and the reverse primer CTTTCCTCCAGCCCCTGTTC (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate  
15 dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

20 **Example 15**

**Antisense inhibition of SRA mRNA - phosphorothioate oligodeoxynucleotides**

In accordance with the present invention, a series of oligonucleotides were designed to target different regions  
25 of four sequences encoding human SRA RNA, incorporated herein as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers on the target sequence to which the oligonucleotide binds. All  
30 compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on SRA mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two  
35 experiments. If present, "N.D." indicates "no data".

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Table 1  
Inhibition of SRA mRNA levels by phosphorothioate  
oligodeoxynucleotides

5	ISIS#	TARGET (SEQ ID)	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
	30138	1	1	cgagcgcctgggtccgcc	52	11
	30139	1	18	accctcttggtaaggcagc	0	12
	30140	1	77	gaggcccaggagaagtct	38	13
	30141	1	114	ggagccttacttgaagga	46	14
10	30142	1	139	accactccccacagggtgg	0	15
	30143	1	148	agaggcaggaccactccc	8	16
	30144	1	158	gctccacgcccagaggcag	51	17
	30145	1	168	aaaccttgtggctccacg	82	18
	30146	1	178	ctcgactggaaaacttgt	37	19
15	30147	1	188	gagcctcagactcgactg	35	20
	30148	1	199	ctccatcagtcgagcctc	0	21
	30149	1	208	cagcacatccatccatcag	39	22
	30150	1	220	ttccaaaggctctcagcac	51	23
	30151	1	229	caatgcctgttccaaagg	9	24
20	30152	1	240	cggcagtcttccaatgcc	0	25
	30153	1	250	tgtgtggccacggcagtc	53	26
	30154	1	261	acctgcttccttgtgtgg	68	27
	30155	1	272	tgtcatcacatacctgct	24	28
	30156	1	282	cgtcggctgtatgtcatca	39	29
25	30157	1	292	cagtgccaggcgtcggct	34	30
	30158	1	303	tgttcctgcagcagtgcc	61	31
	30159	1	314	ctccagcccactgttcct	0	32
	30160	1	325	tgacaactttccatccagc	85	33
	30161	1	336	tttacaggtattgacaac	0	34
30	30162	1	347	ccattctcttctttacag	40	35
	30163	1	359	gcaccagttagagccattc	70	36
	30164	1	370	tgaaagctttgcaccag	36	37
	30165	1	382	ccaccgggtggcttggaaag	15	38
	30166	1	394	atctgctgcgtcccacccg	10	39
35	30167	1	403	gtggatgtcatctgctgc	0	40
	30168	1	414	atgaggggagcgggtggatg	0	41
	30169	1	425	catggtcaaccatgaggg	0	42
	30170	1	438	ctgacactcagtcacatgg	0	43
	30171	1	450	accatccactgactgacc	0	44
40	30172	1	462	cttttaactcctaccatc	57	45
	30173	1	478	cttttctgcaattaaatct	39	46
	30174	1	491	aaaacagactcctctttt	46	47
	30175	1	507	ttggctgcctcctctgaa	42	48

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30176	1	542	ggttcttctcagctgtgg	51	49	
30177	1	585	ggaaccgaggattatgaa	32	50	
30218	2	1	ccgcgttccttggcg	6	51	
30219	2	7	ttccagccgcgttccttgc	0	52	
5	30220	2	22	aactgcggcggtcggttc	0	53
	30221	2	47	ggcctgggtctgcagcccc	6	54
	30222	2	59	cctgggtccgcggcctg	14	55
	30223	2	75	tggtaaggcagcgagcgcc	26	56
	30224	2	139	ggcccaggagaagtctct	0	57
10	30225	2	167	tgaaggagggtggaggccc	0	58
	30226	2	204	gaccactccccacaggtg	0	59
	30227	2	238	actgggaaaacttgtgggc	0	60
	30229	2	273	tcagcacatcctccatca	3	61
	30230	2	310	tggccacggcagtcttcc	48	62
15	30231	2	324	cctgcttccttgtgtggc	34	63
	30232	2	345	gtcggctgtatgtcatcac	0	64
	30233	2	383	cttcctccagccccactg	64	65
	30236	2	456	ctgctgcgtcccacccgt	79	66
	30237	2	471	agcggtggtatgtcatctg	35	67
20	30238	2	492	tcacatggtcaaccatga	7	68
	30239	2	525	ttttaactcctaccatcc	0	69
	30240	2	540	tttctgcaattaatcttt	0	70
	30241	2	561	cctctgaaaacagactcc	29	71
	30242	2	581	tttctcttcattggctgc	62	72
25	30243	2	599	ctcagctgtggctgcaga	0	73
	30244	2	615	gtatggtatggttttct	6	74
	30245	2	629	ctgctgaaaggcctggtat	23	75
	30246	2	637	tatgaaggcctgctggaaag	36	76
	30247	2	667	ggtgtccggtgagtcgtgg	50	77
30	30248	2	686	tctccaaggcataggaga	20	78
	30249	2	696	tgacagaaggctccaag	19	79
	30250	2	705	ggagccaagtgcacagaag	14	80
	30251	2	720	ggtgtggtaagaaggaga	0	81
	30252	2	745	gggtcaggcccagtggga	0	82
35	30253	2	759	cttccctcataggtgggt	0	83
	30254	2	779	ctctggcccagggtggac	45	84
	30255	2	792	acacatgaactccctctg	23	85
	30256	2	808	atgcatgttatgagtaac	0	86
	30257	2	835	tttcaccgcagagatgtt	0	87
40	30371	3	56	gagggtccatactaagcg	47	88
	30372	3	103	gaggcccggaaactccact	11	89
	30373	3	140	ttccttggcgctgcggga	28	90
	30374	3	174	cgtatgagaactgcggcg	0	91

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	30375	3	284	aggcccagggagaagtctc	39	92
	30376	3	361	acgccagaggcaggacca	45	93
	30377	3	400	agtgcgacgttcagactcg	28	94
	30378	3	473	acataacctgcttcattgt	0	95
5	30379	3	492	ggcgtcggctgtatgtcat	58	96
	30380	3	509	tccctgcagcagtgccag	55	97
	30381	3	529	aactttcctccagcccac	79	98
	30382	3	547	ttctttacaggtattgac	0	99
	30383	3	581	gcttcaaagctttgcac	0	100
10	30384	3	601	tctgctgcgtcccaccgg	68	101
	30385	3	620	gaggggacgggtggatgtc	52	102
	30386	3	640	tcagtcacatggtaacc	50	103
	30387	3	663	ctcctaccatccactgac	54	104
	30388	3	683	ttctgcaattaatcttt	41	105
15	30389	3	702	ctgaaaacacagactcctct	44	106
	30390	3	720	cttcattggctgcctcct	80	107
	30391	3	756	tggtatggttcttctcag	42	108
	30392	3	772	tgctggaaggcttggtatg	72	109
	30393	3	791	aaccgaggattatgaagc	43	110
20	30394	3	827	ggcacccgaaaggagatgg	57	111
	30395	3	847	gacaccagaggacttagc	27	112
	30396	3	867	cagggagcaggcgactcg	65	113
	30397	3	885	cagaggtttcagataca	65	114
	30398	3	903	tgaatggagaaggagaa	25	115
25	30399	3	922	ccagcagatcccttcctg	0	116
	30400	3	944	gtaggcagtagtctgact	58	117
	30401	3	977	ggatgcttggctcagtcta	0	118
	30402	3	992	gctccgcccagcacagga	63	119
	30403	3	1007	catccaaacacagctgct	61	120
30	30404	3	1027	ttctcatgctgaaatgaa	26	121
	30309	4	269	cccggttccacgtacagc	37	122
	30310	4	288	ccagccgcgttccttgg	31	123
	30311	4	323	gtctgcagccccgtatgag	0	124
	30312	4	339	gggtccgcggcctgggt	0	125
35	30313	4	359	ttggtaaggcagcgagcgc	44	126
	30314	4	448	aaggagggtggaggccca	28	127
	30315	4	462	gggagccttacttgaagg	12	128
	30316	4	498	gccagaggcaggaccact	28	129
	30317	4	549	atcctccatcagtcgagc	0	130
40	30318	4	566	tccaaagggtctcagcaca	74	131
	30319	4	600	ccttgtgtggccacggca	57	132
	30320	4	616	catcacatacctgcttcc	32	133
	30321	4	635	gccaggcgctggctgatg	62	134

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30322	4	669	caactttcctccagccca	59	135
30323	4	703	ccagtagagccattctct	17	136
30324	4	738	tgcgtcgccccaccggtg	73	137
30328	4	739	gctgtggctgcagatttc	52	138
5 30325	4	773	acatggtcaaccatgagg	46	139
30326	4	807	ttaactcctaccatcca	11	140
30327	4	842	tctgaaaacagactcctc	1	141
30329	4	913	cctgctggaagcctggta	0	142
30330	4	962	atttgaggagatggtgtc	6	143
10 30331	4	997	cttcctttgttgggg	0	144
30332	4	1032	gtatctcaagggtgatac	40	145
30333	4	1067	ccccactcaatgtgggtc	69	146

As shown in Table 1, SEQ ID NOS 11, 13, 14, 17, 18,  
 15 19, 20, 22, 23, 26, 27, 29, 30, 31, 33, 35, 36, 37, 45, 46,  
 47, 48, 49, 50, 62, 63, 65, 66, 67, 72, 76, 77, 84, 88, 92,  
 93, 96, 97, 98, 101, 102, 103, 104, 105, 106, 107, 108,  
 109, 110, 111, 113, 114, 117, 119, 120, 122, 123, 126, 131,  
 132, 133, 134, 135, 137, 138, 139, 145 and 146 demonstrated  
 20 at least 30% inhibition of SRA mRNA levels in this assay  
 and are therefore preferred.

**Example 16:**

**Antisense inhibition of SRA - phosphorothioate 2'-MOE  
gapmer oligonucleotides**

25 In accordance with the present invention, a second series of oligonucleotides targeted to human SRA were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the target sequence, to which the  
 30 oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-  
 35 nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout

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the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from 5 two experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of SRA mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

10	ISIS#	TARGET	TARGET	SEQUENCE	%	SEQ ID NO.
		(SEQ ID)	SITE		Inhibition	
	30178	1	1	cgagcgcctgggtccgcc	54	11
	30179	1	18	accctcttggtaaggcagc	0	12
	30180	1	77	gaggcccaggagaagtct	44	13
15	30181	1	114	ggagccttacttgaagga	38	14
	30182	1	139	accactccccacagggtgg	49	15
	30183	1	148	agaggcaggaccactccc	0	16
	30184	1	158	gctccacgcccagaggcag	44	17
	30185	1	168	aaaccttgtggctccacg	48	18
20	30186	1	178	ctcgactggaaaacttgt	39	19
	30187	1	188	gagcctcagactcgactg	24	20
	30188	1	199	ctccatcagtcgagcctc	28	21
	30189	1	208	cagcacatcctccatcag	0	22
	30190	1	220	ttccaaaggctcagcac	44	23
25	30191	1	229	caatgcctgttccaaagg	29	24
	30192	1	240	cggcagtcttccaatgcc	0	25
	30193	1	250	tgtgtggccacggcagtc	0	26
	30194	1	261	acctgcttccttggtgg	0	27
	30195	1	272	tgtcatcacatacctgct	0	28
30	30196	1	282	cgtcggctgatgtcatca	57	29
	30197	1	292	cagtgccaggcgctcgct	39	30
	30198	1	303	tgttcctgcagcagtgcc	0	31
	30199	1	314	ctccagcccactgttccct	27	32
	30200	1	325	tgacaactttcctccagc	18	33
35	30201	1	336	tttacaggtattgacaac	0	34
	30202	1	347	ccattctcttcttacag	29	35
	30203	1	359	gcaccagtagagccattc	22	36
	30204	1	370	tgaaagctcttgcaccag	27	37
	30205	1	382	ccaccggtgcttgaaag	39	38
40	30206	1	394	atctgctgcgtcccaccg	22	39
	30207	1	403	gtggatgtcatctgctgc	63	40

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30208	1	414	atgaggggagcggtggatg	43	41	
30209	1	425	catggtcaaccatgaggg	56	42	
30210	1	438	ctgacacctagtcacatgg	41	43	
30211	1	450	accatccactgactgacc	0	44	
5	30212	1	462	cttttaactcctaccatc	42	45
30213	1	478	cttttctgcaattaatct	0	46	
30214	1	491	aaaacagactcctctttt	31	47	
30215	1	507	ttggctgcctcctctgaa	68	48	
30216	1	542	ggttttctcagctgtgg	0	49	
10	30217	1	585	ggaaccgaggattatgaa	71	50
30258	2	1	ccgcgttccttgttgccg	53	51	
30259	2	7	ttccagccgcgttccttg	0	52	
30260	2	22	aactgcggcgggtcgttc	0	53	
30261	2	47	ggcctgggtctgcagccc	37	54	
15	30262	2	59	cctgggtccggcggcctg	27	55
30263	2	75	tggtaagcagcgagcgcc	0	56	
30264	2	139	ggcccaggagaagtctct	43	57	
30265	2	167	tgaaggaggtggaggccc	29	58	
30266	2	204	gaccactccccacaggtg	28	59	
20	30267	2	238	actggaaacttgtggc	7	60
30269	2	273	tcagcacatcctccatca	0	61	
30270	2	310	tggccacggcagtcttcc	50	62	
30271	2	324	cctgcttccttgtgtggc	59	63	
30272	2	345	gtcggctgatgtcatcac	38	64	
25	30273	2	383	cttcctccagcccactg	0	65
30276	2	456	ctgctgcgtcccaccggt	83	66	
30277	2	471	agcgggtggatgtcatctg	67	67	
30278	2	492	tcacatggtcaaccatga	62	68	
30279	2	525	ttttaactcctaccatcc	0	69	
30	30280	2	540	tttctgcaattaatcttt	18	70
30281	2	561	cctctgaaaaacagactcc	3	71	
30282	2	581	tttctcttcattggctgc	77	72	
30283	2	599	ctcagctgtggctgcaga	55	73	
30284	2	615	gtatggtatggttcttct	56	74	
35	30285	2	629	ctgctggaaaggctggtat	51	75
30286	2	637	tatgaaggcctgctggaaag	22	76	
30287	2	667	ggtgtccgggtgagtcgtgg	0	77	
30288	2	686	tctccaaggcataggaga	0	78	
30289	2	696	tgacagaaggctccaag	73	79	
40	30290	2	705	ggagccaagtgcacagaag	27	80
30291	2	720	ggtgtggtaagaaggga	34	81	
30292	2	745	gggtcaggcccagtggga	0	82	
30293	2	759	cttcctcatagggtgggt	51	83	

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30294	2	779	ctctggcccaggtggac	50	84
30295	2	792	acacatgaactccctctg	41	85
30296	2	808	atgcatttatcgataac	70	86
30297	2	835	tttcaccgcagagatgtt	35	87
5 30407	3	56	gagggtccatactaagcg	70	88
30408	3	103	gaggccccgaaactccact	53	89
30409	3	140	ttcccttgtgcctgcgga	69	90
30410	3	174	cgtatgagaactgcggcg	88	91
30411	3	284	aggcccagggagaagtctc	79	92
10 30412	3	361	acgccagaggcaggacca	57	93
30413	3	400	agtcgagcctcagactcg	71	94
30414	3	473	acatacctgcttccttgc	22	95
30415	3	492	ggcgtcggctgatgtcat	73	96
30416	3	509	ttcctgcagcagtgccag	85	97
15 30417	3	529	aaccttccctccagccac	69	98
30418	3	547	ttctttacaggtattgac	70	99
30419	3	581	gcttgaaagctttgcac	77	100
30420	3	601	tctgctgcgtcccaccgg	82	101
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20 30422	3	640	tcagtcacatggtaacc	78	103
30423	3	663	ctcctaccatccactgac	56	104
30424	3	683	ttctgcaattaatcttt	67	105
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30426	3	720	cttcattggctgcctcct	81	107
25 30427	3	756	tggtatggttcttcag	90	108
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30429	3	791	aaccgaggattatgaagc	78	110
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30 30432	3	867	cagggagcagggcagtcg	43	113
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30436	3	944	gtaggcagtagtctgact	75	117
35 30437	3	977	ggatgcttgcgtctca	21	118
30438	3	992	gctccgcccagcacagga	79	119
30439	3	1007	catccaaacacagctgct	72	120
30440	3	1027	ttctcatgcgtaaatgaa	62	121
30345	4	269	cccggttacgtacagc	57	122
40 30346	4	288	ccagccgcgttcctgtt	73	123
30347	4	323	gtctgcagccgtatgag	36	124
30348	4	339	gggtccgcggcctgggt	23	125
30349	4	359	ttggtaagcagcgagcgc	83	126

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30350	4	448	aaggaggtggaggcccc	52	127
30351	4	462	gggagccttacttgaagg	3	128
30352	4	498	gccagaggcaggaccact	82	129
30353	4	549	atcctccatcagtcgagc	0	130
5 30354	4	566	tccaaaggcttcagcaca	80	131
30355	4	600	ccttgtgtggccacggca	82	132
30356	4	616	catcacatacctgcttcc	35	133
30357	4	635	gccaggcgtcggtgatg	64	134
30358	4	669	caactttcctccagccca	41	135
10 30359	4	703	ccagtagagccattctct	70	136
30360	4	738	tgctgcgtcccaccggtg	87	137
30364	4	739	gctgtggctgcagattc	76	138
30361	4	773	acatggtcaaccatgagg	87	139
30362	4	807	tttaactcctaccatcca	14	140
15 30363	4	842	tctgaaaacagactcctc	38	141
30365	4	913	cctgctggaaggcctggta	74	142
30366	4	962	atttggaggatgggtgc	56	143
30367	4	997	cttcctttgttggaggg	60	144
30368	4	1032	gtatctcaagggtgatac	72	145
20 30369	4	1067	cccaactcaatgtggttc	81	146

As shown in Table 2, SEQ ID NOS 11, 13, 14, 15, 17, 18, 19, 23, 29, 30, 38, 40, 41, 42, 43, 45, 47, 48, 50, 51, 54, 57, 62, 63, 64, 66, 67, 68, 72, 73, 74, 75, 79, 81, 83, 25 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 113, 114, 115, 116, 117, 119, 120, 121, 122, 123, 124, 126, 127, 129, 131, 132, 133, 134, 135, 136, 137, 138, 139, 141, 142, 143, 144, 145 and 146 demonstrated at least 30% inhibition of SRA expression in this experiment and are therefore preferred.

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**What is claimed is:**

1. An antisense compound 8 to 30 nucleobases in length targeted to human SRA or a nucleic acid molecule encoding human SRA, wherein said antisense compound specifically hybridizes with and decreases the function or amount of human  
5 SRA.
2. The antisense compound of claim 1 which is an antisense oligonucleotide.
3. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO:  
10 11, 13, 14, 15, 17, 18, 19, 20, 22, 23, 26, 27, 29, 30, 31,  
33, 35, 36, 37, 38, 40, 41, 42, 43, 45, 46, 47, 48, 49, 50,  
62, 63, 64, 65, 66, 67, 68, 72, 73, 74, 75, 76, 77, 79, 81,  
83, 84, 85, 86, 87, 51, 54, 57, 88, 89, 90, 91, 92, 93, 94,  
96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108,  
15 109, 110, 111, 113, 114, 115, 116, 117, 119, 120, 121, 122,  
123, 124, 126, 127, 129, 131, 132, 133, 134, 135, 136, 137,  
139, 141, 138, 142, 143, 144, 145 or 146.
4. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID  
20 NO: 11, 13, 14, 17, 18, 19, 23, 29, 30, 45, 47, 48, 50, 62,  
63, 66, 67, 72, 84, 88, 92, 93, 96, 97, 98, 101, 102, 103,  
104, 105, 106, 107, 108, 109, 110, 111, 113, 114, 117, 119,  
120, 122, 123, 126, 131, 132, 133, 134, 135, 137, 139, 138,  
145 or 146.
- 25 5. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
6. The antisense compound of claim 5 wherein the modified internucleoside linkage is a phosphorothioate  
30 linkage.
7. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

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8. The antisense compound of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

9. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

10. The antisense compound of claim 9 wherein the modified nucleobase is a 5-methylcytosine.

11. The antisense compound of claim 1 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

10 12. A composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the antisense 15 compound is an antisense oligonucleotide.

15. A method of decreasing the function or amount of SRA in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that the function or amount of SRA is inhibited.

20 16. A method of treating a human having a disease or condition associated with SRA comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that the function or amount of SRA is inhibited.

25 17. The method of claim 16 wherein the disease or condition is associated with steroid hormones.

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U.S. CALENDAR NO. 00-1140

## SEQUENCE LISTING

<110> Lex M. Cowser  
 C. Frank Bennett  
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 ISIS PHARMACEUTICALS, INC.  
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<120> ANTISENSE MODULATION OF SRA EXPRESSION

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Docket No.  
RTSP-0153

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

### Antisense Modulation of SRA Expression

the specification of which

(check one)

is attached hereto.

was filed on 23 March 2000 as United States Application No. or PCT International

Application Number PCT/US00/07634

and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

### Prior Foreign Application(s)

### Priority Not Claimed

(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

---

(Application Serial No.)

---

(Filing Date)

---

(Application Serial No.)

---

(Filing Date)

---

(Application Serial No.)

---

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

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**09/280,409**

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**March 29, 1999**

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**Patented**

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(Application Serial No.)

---

(Filing Date)

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(Status)

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(patented, pending, abandoned)

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(Application Serial No.)

---

(Filing Date)

---

(Status)

---

(patented, pending, abandoned)

---

(Application Serial No.)

---

(Filing Date)

---

(Status)

---

(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)



26259

PATENT TRADEMARK OFFICE

7-  
 Herb Boswell, Reg. No. 27,311  
 Laurel Spear Bernstein, Reg. No. 37,280  
 Robert S. Andrews, Reg. No. 44,508  
 April Logan, Reg. No. 33,950  
 Neil S. Bartfeld, Reg. No. 39,901  
 Kenneth H. Tarbet, Reg. No. 43,181  
 Donna T. Ward, Reg. No. P48,271  
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 2292 Faraday Avenue  
 Carlsbad, California 92008

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Direct Telephone Calls to: (*name and telephone number*)  
 Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

Full name of sole or first inventor <u>Lex M. Cowser</u>	Date 8/23/01
Sole or first inventor's signature 	
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Citizenship <u>US</u>	
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San Mateo, California 94402	

Full name of second inventor, if any <u>C. Frank Bennett</u>	Date 9-11-01
Second inventor's signature 	
Residence <u>Carlsbad, California</u>	
Citizenship <u>US</u>	
Post Office Address <u>1347 Cassins Street</u>	
Carlsbad, California 92008	

Full name of third inventor, if any <b>Burt W. O'Malley</b>	Date
Third inventor's signature	
Residence <b>Houston, Texas</b>	
Citizenship <b>US</b>	
Post Office Address <b>639 Ramblewood</b>	
<b>Houston, Texas 77030</b>	

Full name of fourth inventor, if any	Date
Fourth inventor's signature	
Residence	
Citizenship	
Post Office Address	

Full name of fifth inventor, if any	Date
Fifth inventor's signature	
Residence	
Citizenship	
Post Office Address	

Full name of sixth inventor, if any	Date
Sixth inventor's signature	
Residence	
Citizenship	
Post Office Address	

Docket No.

RTSP-0153

# Declaration and Power of Attorney For Patent Application

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Prior Foreign Application(s)

Priority Not Claimed

(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>
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(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

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09/280,409	March 29, 1999	Patented
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*



26259

PATENT TRADEMARK OFFICE

Herb Boswell, Reg. No. 27,311  
 Laurel Spear Bernstein, Reg. No. 37,280  
 Robert S. Andrews, Reg. No. 44,508  
 April Logan, Reg. No. 33,950  
 Neil S. Bartfeld, Reg. No. 39,901  
 Kenneth H. Tarbet, Reg. No. 43,181  
 Donna T. Ward, Reg. No. P48,271  
 of the firm  
 ISIS Pharmaceuticals, Inc.-Carlsbad Research Center  
 2292 Faraday Avenue  
 Carlsbad, California 92008

Send Correspondence to:

Direct Telephone Calls to: *(name and telephone number)*

Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

Full name of sole or first inventor <b>Lex M. Cowsert</b>	Date
Sole or first inventor's signature	
Residence <b>San Mateo, California</b>	
Citizenship <b>US</b>	
Post Office Address <b>1299 Parrott Drive</b>	
<b>San Mateo, California 94402</b>	

Full name of second inventor, if any <b>C. Frank Bennett</b>	Date
Second inventor's signature	
Residence <b>Carlsbad, California</b>	
Citizenship <b>US</b>	
Post Office Address <b>1347 Cassins Street</b>	
<b>Carlsbad, California 92008</b>	

3rd

Full name of third inventor, if any <u>Burt W. O'Malley</u>	Date 9/7/01
Third inventor's signature <i>Burt W. O'Malley</i>	Date
Residence <u>Houston, Texas</u>	
Citizenship <u>US</u>	
Post Office Address <u>639 Ramblewood</u>	
<b>Houston, Texas 77030</b>	

09522514 - PTO-951 (Rev. 6-95)

Full name of fourth inventor, if any	Date
Fourth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of fifth inventor, if any	Date
Fifth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of sixth inventor, if any	Date
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	